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(54) Title: METHOD OF DETECTING NEOPLASTIC, HYPERPLASTIC, CYTOLOGICALLY DYSPLASTIC AND/OR PREMALIGNANT CELLULAR GROWTH OR PROLIFERATION

(57) Abstract: Disclosed is a method of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject that involves collecting a sample from the human subject of a bodily substance, such as urine, blood, semen, saliva, mucus, feces, or cellular material, which contains the human subject's nucleic acid or protein. Expression and/or overexpression of nucleic acid or protein products of *survivin* gene, detected in the bodily substance, is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation. Detection is through amplification by molecular biological techniques of *survivin*-specific nucleic acids and analysis of the amplification products, or by immunochemical assay for Survivin protein. Embodiments of the method are disclosed for detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation of the human urinary tract, such as a urinary tract neoplasm. Also disclosed are *survivin* gene-specific primers and probes, primer sets, and diagnostic kits containing them.

METHOD OF DETECTING NEOPLASTIC, HYPERPLASTIC, CYTOLOGICALLY DYSPLASTIC AND/OR PREMALIGNANT CELLULAR GROWTH OR PROLIFERATION

BACKGROUND OF THE INVENTION

Throughout the application various publications are referenced in parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in the application in order to more fully describe the state of the art to which this invention pertains.

1. THE FIELD OF THE INVENTION

This invention relates to the medical arts. In particular, it relates to a diagnostic method for detecting abnormal cellular proliferations including neoplasms.

2. DISCUSSION OF THE RELATED ART

Cancer remains second only to cardiovascular disease as a cause of death in the United States, eventually affecting about a third of all individuals. Over the last few decades, five-year relative survival rates for cancer patients have improved as a result of new therapies and diagnostics. Critical to successful treatment is the early detection of malignancies, but some forms, including urinary tract neoplasms, are less easily detected because clinical symptoms may not appear until relatively late in the development of the disease.

Urinary tract cancers include renal cell carcinoma, bladder carcinoma, nephroblastoma, ureteral and renal pelvic tumors, including transitional cell cancer of the renal pelvis. In the United States, over 75,000 new cases are diagnosed annually, and about 20,000 individuals die. (M.B. Garnick and B.M. Brenner, *Tumors of the Urinary Tract*, In: *Harrison's Principles of Internal Medicine*, 13th Edition, Isselbacher *et al.*, eds., McGraw-Hill, pp. 1336-39 [1994]). Patients most commonly first present with hematuria (bloody urine), but other first signs include pelvic or flank pain, dysuria, or changes in urinary frequency or urgency. By the time the first signs of disease appear a urinary tract cancer may have already begun to invade nearby tissues or may have metastasized to form tumors in distant tissues. Consequently, for many patients newly diagnosed with a urinary tract cancer, the long-term prognosis is poor. In addition, benign neoplasms can cause persistent hematuria, can lead to the loss of kidney function, and like the renal oncocytoma, can undergo life-threatening malignant degeneration. Thus, there has been a definite need

to develop a diagnostic test for benign and malignant neoplasms of the urinary tract that can be done routinely, non-invasively, and cheaply in order to screen large numbers of people for urinary tract cancers before symptoms have appeared.

Most efforts in this direction have focused on detecting the presence of protein markers characteristic of particular neoplasms. For example, a method for diagnosing bladder cancer involved analyzing a urine sample for the presence of a bladder cancer-specific 180 kD protein in a complex with gamma globulin. (Zhau *et al.*, *Unique protein marker for bladder cancer*, U.S. Patent Nos. 5,221,612 and 5,359,031). A different diagnostic/prognostic method for squamous cell carcinoma or urinary tract cancer involved the detection in a urine or a bladder wash sample of comparatively elevated levels of a 90 to115 kD ectodomain portion of the epidermal growth factor (EGFr). (Harvey *et al.*, *Method of cancer detection*, U.S. Patent No. 5,344,760). Other methods of screening for human bladder cancer involved determining an increased level of scatter factor (a basic heparin-binding non-proteolytic glycoprotein, structurally related to plasminogen, and consisting of a 58 kD and a 31 kD subunit) in a urine sample (Goldberg *et al.*, *Method of diagnosing bladder cancer*, U.S. Patent No. 5,656,443), or detecting increased expression of gp78-hAMFR protein on the surface of biopsied bladder cells (Raz *et al.*, *Method of determining metastatic potential of bladder tumor cells*, U.S. Patent No. 5,382,521). Detection of immunoreactive complexes from a urine sample that comprise basement membrane components and polypeptides of various sizes was employed in a method of determining the invasiveness of a bladder tumor. (Houghton *et al.*, *Methods for Determining the invasiveness of a bladder cancer*, U.S. Patent No. 5,541,076).

The BTA stat test (Bard Diagnostic Sciences, Redmond WA) is a commercially available immunochromatographic assay for human complement factor H-related protein (hCFHrp), expressed by some bladder cancer cells, but not by other epithelial cells. The BTA stat test was reportedly able to detect about two-thirds of recurrent bladder cancers. (M.F. Sarosdy *et al.*, *Improved detection of recurrent bladder cancer using the Bard BTA stat test*, Urology 50(3):349-53 [1997]). This is more sensitive than cytological analysis of voided urine or the bladder tumor antigen (BTA) test. A nuclear matrix protein 22 (NMP22) ELISA test kit was sensitive to about 80% of cases of urothelial cancer. (N. Miyanaga *et al.*, *Clinical evaluation of nuclear matrix protein 22 [NMP22] in urine as a novel marker for urothelial cancer*, Eur. Urol. 31:163-68 [1997]). But both of these tests were significantly less sensitive to early stage tumors, which

points to a disadvantage of protein marker tests for the screening of undiagnosed and pre-symptomatic urinary tract cancers. Another is the narrow specificity of many protein markers that have been employed.

Telomerase enzyme activity has previously been employed in the detection of cancer of the bladder, but telomerase enzyme activity was detectable in only 7% of bladder carcinoma patients tested and telomerase riboprotein was detected in only about 80%, with significant numbers of false positives. (M. Müller *et al.*, *Comparison of human telomerase RNA and telomerase activity in urine for diagnosis of bladder cancer*, Clin. Cancer Res. 4:1949-54 [1998]). Thus, telomerase activity is not a satisfactory test for early stage tumors.

While the sensitivity of immunoassay systems, enzyme assays, and other protein detection methods is limited, technology for nucleic acid amplification, such as polymerase chain reaction (PCR)-based or ligase chain reaction (LCR)-based technology is well established as a highly sensitive means of amplifying and detecting the presence of extremely minute quantities of specific nucleic acid markers. Nucleic acid markers have been used in a method of detecting ductal carcinoma in pre-invasive cancerous breast tissue (Holt *et al.*, *Method of detection and diagnosis of pre-invasive cancer*, U.S. Patent No. 5,677,125) or liver cancer (Demetriou *et al.*, *Method and probe for detection of gene associated with liver neoplastic disease*, U.S. Patent No. 5,866,329). A p53 gene mutation has been identified in about half of all human tumor types and has been suggested as a prognostic factor for already detected cases of esophageal squamous cell carcinoma (S. Kobayashi *et al.*, *The p53 gene mutation is of prognostic value in esophageal squamous cell carcinoma patients in unified stages of curability*, Am. J. Surg. 177(6):497-502 [1999]). A genetic marker useful for detecting diverse neoplasms, including urinary tract cancers, has been needed. A gene that is expressed in a wide variety of cancer cell lines, including a renal cancer cell line, is the *survivin* gene. (I. Tamm *et al.*, *IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas(CD95), Bax, caspases, and anticancer drugs*, Cancer Res. 58(23):5315-20 [1998]; G. Ambrosini *et al.*, *A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma*, Nat. Med. 3(8):917-21 [1997]). Survivin is a protein belonging to the inhibitor of apoptosis protein (IAP) family (E.C. LaCasse *et al.*, *The inhibitors of apoptosis [IAPs] and their emerging role in cancer*, Oncogene 17(25):3247-59 [1998]; Altieri *et al.*, *Survivin, a cellular protein that inhibits cellular apoptosis, and its modulation*, WO9822589A2). Survivin is thought to contribute to tissue homeostasis and differentiation during mammalian embryonic and

fetal development; a single 16.5 kD Survivin was detected in human fetal lung, liver, heart, kidney, and gastrointestinal tract. (C. Adida *et al.*, *Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation*, Am. J. Pathol. 152(1):43-49 [1998]). In adults, Survivin may be expressed during cell development, for example, in epidermal basal cells, and in many human cancer cells, but not in normal non-proliferating terminally differentiated adult cell populations. (E.g., D. Grossman *et al.*, *Expression of apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line*, Lab. Invest. 79(9):1121-26 [1999]; G. Ambrosini *et al.*, *Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting*, J. Biol. Chem. 273(18):11177-82 [1998]; C.D. Lu *et al.*, *Expression of a novel anti-apoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas*, Cancer Res. 58(9):1808-12 [1998]; Y. Saitoh *et al.*, *Analysis of Bcl-2, Bax, and survivin genes in uterine cancer*, Int. J. Oncol. 15(1):137-41 [1999]; C. Chiodino *et al.*, *Communication: expression of the novel inhibitor of apoptosis survivin in normal and neoplastic skin*, J. Invest. Dermatol. 113(3):415-18 [1999]).

Expression of Survivin or other anti-apoptotic proteins, such as Bcl-2, appears to enable cancer cells to better evade native anti-neoplastic immune responses and resist cancer therapies. (Reviewed in M. Jaattela, *Escaping cell death: survival proteins in cancer*, Exp. Cell Res. 248(1):30-43 [1999]). Survivin is able to bind effector cell death proteases caspase-3 and caspase-7 and inhibit cell death in cells exposed to diverse apoptotic signals (Tamm *et al.* [1998]). In cases of colorectal carcinoma, immunohistochemical detection of *survivin* expression, alone or in conjunction with Bcl-2 expression, was demonstrated as a predictive/prognostic parameter of patient survival. (H. Kawasaki *et al.*, *Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer*, Cancer Res. 58(22):5071-74 [1998]). *Survivin* mRNA was present in the urine sediment of patients with positive cytological evaluations for urothelial malignancies. (L.M. Jouben-Steele *et al.*, *Survivin expression in the surveillance of urothelial neoplasia*, Laboratory Investigation 79:99A [1999]).

There remains a definite need for a highly sensitive, accurate, and non-invasive diagnostic test useful in screening for a broad range of abnormal cellular proliferations, including cancerous and benign neoplasms, such as urinary tract neoplasms. It is a desideratum that such a test be capable of detecting early urinary tract neoplasms even before a patient becomes symptomatic. The present inventions provides these and other benefits.

SUMMARY OF THE INVENTION

The present invention relates to a useful method of detecting neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject. The method involves collecting a sample of a bodily substance containing human nucleic acid or protein that originates from cells of the subject. Detecting in the bodily substance the presence or absence of expression of nucleic acid or protein products of human *survivin* gene, which expression is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the human subject. Detection is through amplification by molecular biological techniques of *survivin*-specific nucleic acids and analysis of the amplification products, or alternatively, by immunochemical assay for Survivin protein.

It is a benefit of the inventive method that detection of expression of products of the human *survivin* gene in the sample in accordance with the method alerts the subject's physician that further investigation is warranted employing more expensive and/or invasive procedures (e.g., magnetic resonance imaging, cytoscopy, or tissue biopsies) to pinpoint the location and nature of the abnormal cellular growth or proliferation.

One embodiment of the present invention is a useful method of detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the urinary tract of the human subject, such as, but not limited to, a urinary tract neoplasm. The inventive method is a non-invasive procedure that involves collecting urine from the human subject. The urine sample, or an isolated fraction thereof (e.g., fluid fraction or separated cells), is assayed to detect the level of expression of nucleic acid or protein products of *survivin* gene expression. Detection is through amplification by molecular biological techniques of *survivin*-specific nucleic acids and analysis of the amplification products, or detection is by immunochemical assay for Survivin protein. The inventive method is particularly advantageous in terms of sensitivity and reliability compared with previous methods for detecting urinary tract cancers; *survivin*-specific mRNAs and *Survivin* protein are expressed in early urinary tract cancers, because early cancer cells are frequently in a state of cell division more often than the normal cells found in the same urine sediment. Therefore, it is a benefit of the inventive method that early detection of urinary tract cancers and precancerous conditions are facilitated and prospects for patient survival are thereby enhanced.

The present invention is also related to oligonucleotide primers, probes, primer sets, and kits for detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human. These are useful in practicing the inventive method.

These and other advantages and features of the present invention will be described more fully in a detailed description of the preferred embodiments which follows.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the results of real time PCR applied to the detection of *survivin* oncogene mRNA expression. The graph shows six positive samples at Ct (threshold cycle) 21 (positive at cycle 21), by Taqman probe sequence SRTP (SEQ. ID.NO.:6). The graph was generated with an BioRad iCycler, primers SR1F (SEQ. ID. NO.:1) and SR2R (SEQ. ID. NO.:2); probe SRTP (SEQ. ID. NO.:6) was 5'-FAM and 3'-TAMRA labelled. Amplification conditions were 2 min at 50°C, 10 min at 95°C, then 2 step PCR for 40 cycles at 30 sec at 95°C and 30 sec at 60°C; the probe was used at 200nM final concentration (primers concentrations as described herein). The source of *survivin* was RNA of replicating cell cultures (six strong positives in the graph are: A2=human skin fibroblasts, A3 = human Raji, A4=human MOLT-4, A5=human H9, A6=mouse BNL, A8=human HeLa; negative or weak positives are A1=mouse 3T3 and A7=mouse Hepa 1-6; negative controls are A9= water and A10= water). These results demonstrate prominent amounts of *survivin* expression in the cDNA of human cultures as detected by real time PCR.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a method of detecting neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject, such as in, but not limited to, the urinary tract of the subject. The method involves collecting a sample of a bodily substance derived from the human subject, containing human nucleic acid or protein, and detecting therein the presence or absence of a nucleic acid or protein product of human *survivin* gene expression, which expression is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation.

"Collecting" the sample includes deriving it from the human subject by direct sampling. Any bodily substance containing human nucleic acids from the subject may be collected, i.e., sampled,

gathered, obtained, received and/or transported for the purpose of practicing the method. The sample is collected directly from the human subject's body; preferred and convenient substance for sampling include urine or blood. However, the collected sample can be feces, semen, prostatic fluid, saliva, gastric juice, mucus, synovial fluid, pleural effusion, peritoneal effusion, pericardial effusion, lymph, cerebro-spinal fluid, skin, hair root, vascular epithelium, oral epithelium, vaginal epithelium, cervical epithelium, uterine epithelium, intestinal epithelium, bronchial epithelium, esophageal epithelium, or mesothelium, or other biopsy sample of cellular material from any tissue. Cellular material includes any sample containing human cells, including samples of tissue, expressed tissue fluids (e.g., expressed prostatic fluid), tissue wash or rinsate fluids (e.g., bladder or vaginal wash or rinsate fluids), or the like. Tissue samples that can be collected include, but are not limited to, cell-containing material from the kidney, ureter, bladder, urethra, brain, thyroid, parotid gland, submaxillary gland, sublingual gland, lymph node, bone, cartilage, lung, mediastinum, breast, uterus, ovary, testis, prostate, cervix uteri, endometrium, pancreas, liver, spleen, kidney, adrenal, esophagus, stomach, and/or intestine.

The sample is alternatively derived from cultured human cells, cell-free extracts, or other specimens indirectly derived from a subject's body, as well as from substances taken directly from a subject's body. Samples may be stored before detection methods are applied (for example nucleic acid amplification and/or analysis, or immunochemical detection) by well known storage means that will preserve nucleic acids or proteins in a detectable and/or analyzable condition, such as quick freezing, or a controlled freezing regime, in the presence of a cryoprotectant, for example, dimethyl sulfoxide (DMSO), glycerol, or propanediol-sucrose. Samples may also be pooled before or after storage for purposes of amplifying their *survivin*-specific nucleic acids for analysis and detection, or for purposes of detecting Survivin protein.

The sample is optionally pre-treated by refrigerated or frozen storage overnight, by dilution, by phenol-chloroform extraction, or by other like means, to remove factors that may inhibit various amplification reactions; such as heme-containing pigments or urinary factors. For example, such amplification-inhibitory urinary factors are especially prevalent in the urine of pregnant and non-pregnant females. (E.g., J. Mahony *et al.*, *Urine specimens from pregnant and non-pregnant women inhibitory to amplification of Chlamydia trachomatis nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity*, J. Clin. Microbiol. 36(11):3122-26 [1998]).

Hyperplastic cellular growth or proliferation includes abnormal multiplication or increase in the numbers of normal cells in a normal arrangement in a tissue, for example, as is common in benign prostatic hyperplasia. Cytologically dysplastic and/or premalignant cellular growth or proliferation include increases in cellular numbers of karyotypically abnormal but non-malignant cells within a tissue. Examples include some benign prostatic hyperplasias/dysplasia and cervical hyperplasias/dysplasias.

Neoplastic cellular growth and/or proliferation, i.e., growth of abnormally organized tissue, includes malignant and non-malignant neoplasms. Malignant neoplasms include primary, recurrent, and/or or metastatic cancerous tumors originating in any tissues, for example, carcinomas, sarcomas, lymphomas, mesotheliomas, melanomas, gliomas, nephroblastomas, glioblastomas, oligodendrogliomas, astrocytomas, ependymomas, primitive neuroectodermal tumors, atypical meningiomas, malignant meningiomas, or neuroblastomas, originating in lung, kidney, ureter, bladder, urethra, breast, prostate, testis, skull, brain, spine, thorax, peritoneum, ovary, uterus, stomach, liver, bowel, colon, rectum, bone, lymphatic system, skin, or in any other organ or tissue of the subject.

In some preferred embodiments, the present invention is useful as a method of detecting in a human subject a neoplasm of the urinary tract, which includes the kidneys, ureters, bladder, and urethra. The method involves collecting a urine sample from a human subject. "Collecting" the urine sample also encompasses sampling, receiving, gathering, obtaining, and/or transporting the voided sample or a concentrate, sediment, precipitate, supernatant, filtrate, aspirate, or other fraction thereof. For detection and/or analysis of products of *survivin* gene expression from an isolated cellular fraction of the urine sample, the urine sample is sedimented, typically by centrifugation, or filtration to remove the cells from the liquid fraction, and the mRNA or protein is extracted from the cell sediment or from the cells on the filter by conventional means. Alternatively, the products of *survivin* gene expression can be detected in the liquid (non-cellular) fraction or a filtrate or concentrate thereof. The presence in the sample of a nucleic acid or protein product of *survivin* gene expression is diagnostic for urinary tract neoplasms, as well as for hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the urinary tract.

The urinary tract neoplasm is a benign or non-malignant tumor, for example, an angiomyolipoma, mesoblastic nephroma, or premalignant renal oncocytoma.

Alternatively, the urinary tract neoplasm is a cancerous or malignant tumor. Examples include renal cell carcinoma or hypernephroma, bladder carcinomas, including papillary carcinomas and flat transitional cell carcinomas nephroblastoma (Wilm's tumor), ureteral and renal pelvic tumors, including transitional cell cancer of the renal pelvis. A malignant urinary tract neoplasm can also be a recurrent and/or metastatic tumor of any kind originating in any tissue of the subject's body, as described above.

The nucleotide sequence of the human *survivin* gene, also known as the apoptosis inhibitor 4 (API4) locus, is known (e.g., GenBank Accession U75285). The products of *survivin* gene expression include *survivin*-specific mRNA sequences or Survivin protein. In accordance with the inventive methods, the expression of Survivin protein is optionally detected by immunochemical means, such as, but not limited to, enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IFA), immunoelectrophoresis, immunochromatographic assay or immunohistochemical staining, employing anti-Survivin polyclonal or monoclonal antibodies or antibody fragments, for example Fab, Fab', $F(ab')_2$, or F(v) fragments, that selectively bind Survivin protein. (E.g., C.D. Lu *et al.*, *Expression of a novel anti-apoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas*, Cancer Res. 58(9):1808-12 [1998]; C. Adida *et al.*, *Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation*, Am. J. Pathol. 152(1):43-49 [1998]).

Most preferably, detection of *survivin* mRNAs is accomplished by numerous methods of amplification of *survivin*-specific nucleic acid segments in the form of RNA or cDNA. Before amplification, it is preferable to extract or separate mRNA from DNA in the sample and to amplify nucleic acids remaining in that fraction of the sample separated from the DNA, to avoid false positives that are caused by amplification of contaminating *survivin*-specific genomic DNA in the original specimen. However, there are useful oligonucleotide primer sets that amplify exon-to-exon across an intron of the *survivin* gene, as for example described in Example 2 herein, making extraction or separation of mRNA from genomic DNA unnecessary. The amplifications products, if any, are then analyzed to detect the presence of *survivin* gene-specific amplification products.

If *survivin* gene-specific amplification products are present, the findings are indicative of expression of the *survivin* gene and diagnostic of the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the subject, such as, but not limited to, a urinary tract neoplasm. However, for interpretation of negatives (no *survivin*-

specific amplification products) analysis is preferably carried out following a control amplification of nucleic acids specific for a housekeeping gene, for example, a gene encoding β-actin, phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase, or phosphoglycerate kinase. Only if expression of the housekeeping gene is detected in the sample, is the absence of *survivin* gene expression reliably accepted. With increasing sensitivity of amplification and analysis methods employed, it becomes increasingly preferable to determine the level of *survivin* gene expression relative to expression of a housekeeping gene, in order to better distinguish neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation from the detectable background of normal cellular division. The ratio of *survivin* expression to housekeeping gene expression is determined, for example, by real-time PCR methods or densitometric measurement and analysis of electrophoretic bands after amplification. When the ratio of *survivin* expression to housekeeping gene expression exceeds a normal cell standard range and/or approximates an abnormal (e.g., neoplastic) cell standard range, this indicates overexpression of *survivin* gene product and is diagnostic for neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation.

The mRNAs are amplified by a suitable amplification method. For example, in a preferred embodiment, a reverse transcriptase-mediated polymerase chain reaction (RT-PCR) is employed to amplify *survivin*-specific nucleic acids. Briefly, two enzymes are used in the amplification process, a reverse transcriptase to transcribe *survivin*-specific cDNA from a *survivin*-specific mRNA template in the sample, a thermal resistant DNA polymerase (e.g., *Taq* polymerase), and *survivin*-specific primers to amplify the cDNA to produce *survivin* gene-specific amplification products. The use of limited cycle PCR yields semi-quantitative results. (E.g., Gelfand *et al.*, *Reverse transcription with thermostable DNA polymerase-high tempreature reverse transcription*, U.S. Patent Nos. 5,310,652; 5,322,770; Gelfand *et al.*, *Unconventional nucleotide substitution in temperature selective RT-PCR*, U.S. Patent No. 5,618,703).

In another preferred embodiment of the inventive method, single enzyme RT-PCR is employed to amplify *survivin* gene-specific nucleic acids. Single enzymes now exist to perform both reverse transcription and polymerase functions, in a single reaction. For example, the Perkin Elmer recombinant *Thermus thermophilus* (rTth) enzyme(Roche Molecular), or other similar enzymes, are commercially available.

In a most preferred embodiment, real-time RT-PCR is employed to amplify *survivin* gene-specific nucleic acids. Briefly, this is a quantitative gene analysis based on the ratio of *survivin* gene expression and the expression of a housekeeping gene, i.e., a gene that is expressed at about the same level in normal and abnormal (e.g., malignant) cells, for example, a gene encoding β-actin, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, or phosphoglyceratekinase. The the ratio of the *survivin* and housekeeping genes' expressions is routinely established as a standard for normal and abnormal cells, which standard expression ratio(s) is (are) used for comparison in determining that expression of the *survivin* gene relative to expression of the "housekeeping" gene in a given sample is either "normal" or "increased", the latter indicative of "overexpression" and diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation. In this embodiment, the ratio is the key to diagnosis and constitutes quantitative gene expression analysis. This embodiment utilizes so-called real-time quantitative PCR, carried out with commercially available instruments, such as the Perkin Elmer ABI Prism 7700, the so-called Light Cycler (Roche Molecular), and/or other similar instruments. Optionally, single enzyme RT-PCR technology, for example, employing rTth enzyme, can be used in a real-time PCR system. Preferably, amplification and analysis are carried out in an automated fashion, with automated extraction of mRNA from a urine sediment sample, followed by real-time PCR, and fluorescence detection of amplification products using probes, such as TaqMan or Molecular Beacon probes. Typically, the instrumentation includes software that provides quantitative analytical results during or directly following PCR without further amplification or analytical steps.

In another preferred embodiment, transcription-mediated amplification (TMA) is employed to amplify *survivin* gene-specific nucleic acids. (E.g., K. Kamisango *et al.*, *Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay*, J. Clin. Microbiol. 37(2):310-14 [1999]; M. Hirose *et al.*, *New method to measure telomerase activity by transcription-mediated amplification and hybridization protection assay*, Clin. Chem. 44(12)2446-52 [1998]). Rather than employing RT-PCR for the amplification of a cDNA, TMA uses a probe that recognizes a *survivin*-specific (target sequence) RNA; in subsequent steps, from a promoter sequence built into the probe, an RNA polymerase repetitively transcribes a cDNA intermediate, in effect amplifying the original RNA transcripts and any new copies created, for a

level of sensitivity approaching that of RT-PCR. The reaction takes place isothermally (one temperature), rather than cycling through different temperatures as in PCR.

Other useful amplification methods include a reverse transcriptase-mediated ligase chain reaction (RT-LCR), which has utility similar to RT-PCR. RT-LCR relies on reverse transcriptase to generate cDNA from mRNA, then DNA ligase to join adjacent synthetic oligonucleotides after they have bound the target cDNA.

Most preferably, amplification of a *survivin* gene-specific nucleic acid segment of the subject can be achieved using *survivin* gene-specific oligonucleotide primers and primer sets of the present invention, as described below.

Optionally, high throughput analysis may be achieved by PCR multiplexing techniques well known in the art, employing multiple primer sets, for example primers directed not only to *survivin* gene-specific nucleic acids, but to amplifying expression products of housekeeping genes (controls) or of other potential diagnostic markers (e.g., oncogenes), as well, such as MAG or telomerase, to yield additional diagnostic information. (E.g., Z. Lin *et al.*, *Multiplex genotype determination at a large number of gene loci*, Proc. Natl. Acad. Sci. USA 93(6):2582-87 [1996]; Demetriou *et al.*, *Method and probe for detection of gene associated with liver neoplastic disease*, U.S. Patent No. 5,866,329).

Hybridization analysis is a preferred method of analyzing the amplification products, employing one or more *survivin* gene-specific probe(s) that, under suitable conditions of stringency, hybridize(s) with single stranded *survivin* gene-specific nucleic acid amplification products comprising complementary nucleotide sequences. The amplification products are typically deposited on a substrate, such as a cellulose or nitrocellulose membrane, and then hybridized with labeled *survivin* gene-specific probe(s), optionally after an electrophoresis. Conventional dot blot, Southern, Northern, or fluorescence in situ (FISH) hybridization protocols, *in liquid* hybridization, hybridization protection assays, or other semi-quantitative or quantitative hybridization analysis methods are usefully employed along with the *survivin* gene-specific probes of the present invention. Alternatively, electrophoresis for analyzing amplification products is done rapidly and with high sensitivity by using any of various methods of conventional slab or capillary electrophoresis, with which the practitioner can optionally choose to employ any facilitating means of nucleic acid fragment detection, including, but not limited to, radionuclides, UV-absorbance or laser-induced fluorescence. (K. Keparnik *et al.*, *Fast detection of a (CA)18 microsatellite repeat*

in the IgE receptor gene by capillary electrophoresis with laser-induced fluorescence detection, Electrophoresis 19(2);249-55 [1998]; H. Inoue *et al.*, *Enhanced separation of DNA sequencing products by capillary electrophoresis using a stepwise gradient of electric field strength*, J. Chromatogr. A. 802(1):179-84 [1998]; N.J. Dovichi, *DNA sequencing by capillary electrophoresis*, Electrophoresis 18(12-13):2393-99 [1997]; H. Arakawa *et al.*, *Analysis of single-strand conformation polymorphisms by capillary electrophoresis with laser induced fluorescence detection*, J. Pharm. Biomed. Anal. 15(9-10):1537-44 [1997]; Y. Baba, *Analysis of disease-causing genes and DNA-based drugs by capillary electrophoresis. Towards DNA diagnosis and gene therapy for human diseases*, J. Chromatgr B. Biomed. Appl. 687(2):271-302 [1996]; K.C. Chan *et al.*, *High-speed electrophoretic separation of DNA fragments using a short capillary*, J. Chromatogr B. Biomed. Sci. Appl. 695(1):13-15 [1997]).

Any of diverse fluorescent dyes can optionally be used to label probes or primers or amplification products for ease of analysis, including but not limited to, SYBR Green I, Y1O-PRO-1, thiazole orange, Hex (i.e., 6-carboxy-2',4',7',4,7-hexachlorofluoroscein), pico green, edans, fluorescein, FAM (i.e., 6-carboxyfluorescein), or TET (i.e., 4,7,2',7'-tetrachloro-6-carboxyfluoroscein). (E.g., J. Skeidsvoll and P.M. Ueland, *Analysis of double-stranded DNA by capillary electrophoresis with laser-induced fluorescence detection using the monomeric dye SYBR green I*, Anal. Biochem. 231(20):359-65 [1995]; H. Iwahana *et al.*, *Multiple fluorescence-based PCR-SSCP analysis using internal fluorescent labeling of PCR products*, Biotechniques 21(30:510-14, 516-19 [1996]).

The present invention also relates to *survivin* gene-specific oligonucleotide primers, probes, and primer sets, useful for amplifying and/or analyzing a human *survivin* gene-specific nucleic acid segment, for example, in accordance with the inventive methods. Preferably, a *survivin* gene-specific primer is a *survivin* gene-specific oligonucleotide at least 15 to 30 contiguous nucleotides long, and most preferably 17 to 22 nucleotides long, but primers as short as 7 contiguous nucleotides may be useful for some gene-specific sequences. (E.g., Vincent, J., *et al.*, *Oligonucleonucleotides as short as 7-mers can be used for PCR amplification*, DNA Cell Biol. 13(1):75-82 [1994]). Useful primers include a nucleotide sequence, such as SR1F 5'-TCT TGG AGG GCT GCG CCT GC-3' (forward primer; SEQ. ID. NO.:1); SR2R 5'-AGT CTG GCT CGT TCT CAG TGG-3' (reverse primer; SEQ. ID. NO.:2); or SRP 5'-CAG TGG ATG AAG CCA GCC TC-3' (reverse primer; SEQ. ID. NO.:3). Other useful primers include SRVF1 5' CCC TTT CTC

AAG GAC CAC CG-3' (forward primer; SEQ. ID. NO.:4); and SRVR2 5' ACT GGG CCA AGT CTG GCT CG-3' (reverse primer; SEQ. ID. NO.:5). Another useful primer sequence is SRTP 5'-CCGAGGCTGGCTTCATCCACTGC-3' (forward primer; SEQ. ID. NO.:6). The inventive primers also include a *survivin* gene-specific fragment of any of (SEQ. ID. NOS.:1-6) at least 15 nucleotides long. In addition a sequence complementary to any of (SEQ. ID. NOS.:1-6) or the *survivin* gene-specific fragment, or any *survivin* gene-specific oligonucleotide sequence at least 15 nucleotides long and overlapping at 5 or more contiguous nucleotide positions any of (SEQ. ID. NOS.:1-6) at its 5' or 3' end, or a nucleic acid sequence complementary thereto, is a useful primer sequence.

The inventive probe is preferably 7 to 500 nucleotides long, most preferably 15 to 150 nucleotides long, and comprises, for at least part of its length, a *survivin*-specific nucleotide sequence at least 7 to 15 nucleotides long, such that the probe hybridizes to a survivin-specific single stranded nucleic acid under suitably stringent hybridization conditions. For example, probes comprising the inventive oligonucleotide primer sequences described above (SEQ.ID.NOS.:1-6) can be labeled for use as probes for detecting or analyzing *survivin*-specific nucleic acid amplification products. For example, SRTP (SEQ. ID. NO.:6) is particularly useful as a Taqman type probe in Real Time PCR. (See Figure 1).

The skilled artisan can readily determine other useful *survivin* gene-specific nucleotide sequences for use as primers or probes by conducting a sequence similarity search of a genomics data base, such as the GenBank database of the National Center for Biotechnology Information (NCBI), using a computerized algorithm, such as PowerBLAST, QBLAST, PSI-BLAST, PHI-BLAST, gapped or ungapped BLAST, or the "Align" program through the Baylor College of Medicine server. (E.g., Altchul, S.F., *et al.*, *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*, Nucleic Acids Res. 25(17):3389-402 [1997]; Zhang, J., & Madden, T.L., *PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation*, Genome Res. 7(6):649-56 [1997]; Madden, T.L., *et al.*, *Applications of network BLAST server*, Methods Enzymol. 266:131-41 [1996]; Altschul, S.F., *et al.*, *Basic local alignment search tool*, J. Mol. Biol. 215(3):403-10 [1990]).

Examples of useful sets of oligonucleotide primers for amplifying nucleic acids in accordance with the inventive methods include primer sets comprising (SEQ. ID. NO.:1) and (SEQ. ID. NO.:2) or (SEQ. ID. NO.:3), yielding 81-bp or 58-bp fragments, respectively; or (SEQ. ID.

NO.:4) and (SEQ. ID. NO.:5), which yield a 136-bp fragment. (SEQ. ID. NO.:1) and (SEQ. ID. NO.:5) also comprise a set yielding a 90-bp fragment; and (SEQ. ID. NO.:4) and (SEQ. ID. NO.:2) or (SEQ. ID. NO.:3) comprise sets yielding 127-bp or 104-bp fragments, respectively. The useful primer set SRTP (SEQ. ID. NO.:6) and SR2R (SEQ. ID. NO.:2) yields a 45-bp amplification product, and the useful primer set SRTP (SEQ. ID. NO.:6) and SRVR2 (SEQ. ID. NO.:5) yields a 54-bp fragment. But any set of at least two *survivin* gene-specific primers, as described above, are useful in a set, as long as there is at least one forward and at least one reverse primer, except that SRTP (SEQ. ID. NO.:6) and SRP (SEQ. ID. NO.:3) are not a useful primer set. Exon-to-exon amplification across a *survivin* intron is accomplished by choosing a forward primer and a reverse primer sequence extending, respectively, on opposite sides of the intron.

The present invention is also directed to a diagnostic kit useful for practicing the inventive methods. The kit is an assemblage of materials or components, including at least one of the inventive oligonucleotide primers or probes and/or including the inventive oligonucleotide primer set specific for amplifying and/or analyzing human *survivin* gene-specific sequences, particularly coding sequences, as described above. In addition, the kit contains instructions for using the oligonucleotide primers and/or probes and/or primer set(s) to detect a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation, such as a urinary tract neoplasm. Optionally, the kit also contains other components, such as primers and/or probes for controls (e.g., targeting expression products of housekeeping genes), specimen containers, for example, urine cup(s), or paraphernalia for concentrating, sedimenting, or fractionating a urine sample or a sample of another bodily substance. The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures.

The foregoing descriptions of the methods, probes, primers, primer sets, and kits of the present invention are illustrative and by no means exhaustive. When these features of the present invention are employed in screening for neoplasms, preferably as part of a routine physical examination protocol undertaken on a frequent periodic basis, subjects can benefit from early detection of precancerous or cancerous conditions and can enjoy enhanced prospects for survival and good health after treatment.

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Example 1: Sample preparation

Obtaining Cells from Urine. One milliliter of freshly voided urine or fixed urine with resuspended sediment (urine was fixed with 50% ethanol in water used to dilute urine 50% [v/v]) was centrifuged at 1310 x g for 10 minutes, at room temperature (RT). The supernatant was removed and the pellet was resuspended and extracted in 1.0 mL of RNAzol B (Leedo Medical Labs, Houston) immediately, or was frozen at -70 °C until extraction.

RNA Extraction. Chloroform (0.1 mL) was added to the RNAzol B suspension (above), which was then vortexed for 15 seconds and centrifuged at 16,000 x g (Eppendorf Centrifuge) at 4°C for 15 minutes. The aqueous top layer (about 0.5 mL) was removed with a pipette, and 0.5 mL cold 100% isopropanol was added to it. The isopropanol-diluted aqueous layer was chilled on ice for 10 minutes, then centrifuged at 16,000 x g, at 4°C for 15 minutes. The supernatant was removed and 75% ethanol in DEPC-treated water (RNase free water) was added to the pellet. The resuspended pellet was centrifuged at 16,000 x g at 4°C for 12 minutes, and the alcohol was carefully removed with a pipette and the remaining pellet was dried under vacuum for 10-30 minutes, resuspended in 10 μL DEPC-treated water, and was either used immediately for nucleic acid RT-PCR amplification purposes or frozen at -70°C until used.

Example 2: Amplification of nucleic acids and analysis of amplification products

Reverse Transcription (RT). A Superscript Preamplification kit (GIBCO BRL, Rockville, MD) with random hexamers was used to carry out reverse transcription and prepare for the reverse transcriptase-mediated polymerase chain reaction. Briefly, first strand synthesis was performed with reverse transcriptase, followed by digestion of RNA template with RNase H, in a 20 μL reaction volume.

Polymerase Chain Reaction (PCR) Two microliters of the reverse transcription reaction mixture (above) was taken for the PCR reaction in a final volume of 50 µL PCR reaction mixture containing the following: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$; dNTPs each at 200 µM; primers each at 0.5 µM; 1.5 U *Taq* polymerase (Perkin Elmer, Foster City, CA). Cycling was under the following conditions of temperature and time: denaturation at 94°C for 30 seconds; annealing at 62°C for 30 seconds; extension at 72°C for 60 seconds; final extension for 8 minutes; PCR was carried out for 35-40 cycles. The primer sequences used in the PCR reaction were synthesized by IDT (Coralville, IA). The following primer sequences were used in the amplification:

(a) Set SR1: forward primer, designated SR1F, 5'-TCT TGG AGG GCT GCG CCT GC-3' (SEQ. ID. NO.:1) and reverse primer, designated SR2R, 5'-AGT CTG GCT CGT TCT CAG TGG-3' (SEQ. ID. NO.:2) yielded exon-to-exon amplification across an intron, resulting in an 81-base pair amplification product corresponding to a fragment of the human *survivin* gene. This set of primers (SR1) comprised the primary oligonucleotides for the tests that were conducted; or

(b) a secondary set, used for semi-nested PCR, included SR1F (SEQ. ID. NO.:1) and a reverse primer, designated SRP, 5'-CAG TGG ATG AAG CCA GCC TC-3' (SEQ. ID. NO.:3). This set also yielded exon-to-exon amplification across an intron, resulting in a 58-base pair product; or

(c) Set SRV: forward primer, designated SRVF1, 5' CCC TTT CTC AAG GAC CAC CG-3' (SEQ. ID. NO.:4); and reverse primer, designated SRVR2, 5' ACT GGG CCA AGT CTG GCT CG-3' (SEQ. ID. NO.:5) yielded a 136-base pair product of exon-to-exon amplification across an intron; or

(d) a set including forward primer SR1F (SEQ. ID. NO.:1) and reverse primer SRVR2 (SEQ. ID. NO.:5) yielded exon-to-exon amplification across an intron, resulting in a 90-base pair amplification product; or

(e) a set including forward primer SRVF1 (SEQ. ID. NO.:4) and reverse primer SR2R (SEQ. ID. NO.:2) yielded exon-to-exon amplification across an intron, resulting in a 127-base pair amplification product; or

(f) a set including forward primer SRVF1 (SEQ. ID. NO.:4) and reverse primer SRP (SEQ. ID. NO.:3) yielded exon-to-exon amplification across an intron, resulting in a 104-base pair amplification product.

Detection of PCR amplification products was by electrophoresis (4% agarose gel) with standard size markers; this was followed by ethidium bromide staining, and UV photography for documentation. The *survivin* gene-specificity of PCR amplification products was determined by hybridization (dot blot) with labeled probe and by semi-nested PCR.

Hybridization (dot blot). Five microliters of PCR post-amplification reaction mixture was mixed with 95 μL denaturation solution (0.4 N NaOH; 25 mM EDTA) to a total volume of 100 μL, which was heated to 100°C for 3 minutes, and then chilled on ice. The entire volume of the thus denatured amplification products was applied by vacuum with template to a nylon membrane (blot) and placed in a UV Linker (Stratagene, La Jolla, CA) for cross-linkage of PCR product to the membrane. Twenty units of T4 polynucleotide kinase (New England BioLabs, Beverly MA) was employed for labeling (50 μCi γ-[^{32}P]-ATP; 3000 Ci/mmol; New England Nuclear Life Science Products, Boston, MA) 2 μg of specific probe (SRP, 5' CAG TGG ATG AAG CCA GCC TC-3'; SEQ. ID. NO.:3), according to the manufacturer's directions. Separation of the labeled probe from the isotope was by spin column (Clontech Laboratories, Inc., Palo Alto, CA); Hybrisol II (Oncor, Gaithersburg, MD) was employed for pre-hybridization (60 minutes). Hybridization was carried out using $\geq 5 \times 10^7$ cpm of the labeled probe in 10 mL of Hybrisol II, at 45°C overnight, with agitation in a hybridization chamber (Stratagene, La Jolla, CA); subsequently, the membrane was washed three times with 2x SSC, with 0.1 % SDS for 10 minutes at room temperature, with a final stringency wash in 2x SSC, 0.1 % SDS, for 30 minutes at 60°C; exposure of X-OMAT film (Kodak, Rochester, NY) was for 1 to 3 hours, at -70°C, with intensifying screens.

Semi-Nested PCR. PCR was carried out with the forward (SR1F; SEQ. ID. NO.:1) and reverse (SR2R; SEQ. ID. NO.:2) primers for 35-40 cycles as described above. Five microliters of a 1/100 dilution of the PCR post-amplification reaction mixture was used for semi-nested PCR; the SRP (SEQ. ID. NO.:3) was employed as the reverse primer in a second PCR reaction with the forward SR1F primer (SEQ. ID. NO.:1). PCR was repeated as before, using the same conditions as above, except for an annealing temperature of 55°C, for 25 cycles. Detection of the amplification products was by gel electrophoresis as described above. The PCR amplification product in nested PCR was 58 base pairs in length.

Example 4: Detection of *survivin* nucleic acid transcript in the sample indicates the presence of a neoplastic condition.

Voided urine samples were centrifuged and the urine sediment was analyzed for the presence of *survivin* gene mRNA transcript using RT-PCR as previously described. RT-PCR results were compared to cytologic evaluation for patients who were clinically either screened or followed for neoplasia. Normal (control) urine was collected from males and females 25 to 44 years of age. The presence of *survivin* gene mRNA transcript was detected by analysis of appropriately sized gel electrophoretic bands, and was confirmed by nested PCR and dot blot using the same secondary primer as a probe of the PCR product. There were no false positives by amplification of contaminating DNA, or in normal urine samples. Polymerase chain reaction (PCR) primers were selected and tested to ensure that a PCR amplification product contained only amplification product from the cDNA, not from DNA from the original cellular specimen.

The results in Table 1 demonstrate that *survivin* mRNA is present (PCR[+] reaction) in the urine sediment of patients with positive cytologic evaluations for malignancy (transitional cell carcinoma), but not in normal urine samples. All nine neoplastic cell lines examined also expressed *survivin* mRNA. These cell lines included human cell lines 1 HTB, 5 HTB, Hela, SW480, H9, SK-HEP, MCF7; and murine cell lines 3T3, HEPA 1-6. A non-malignant murine cell line was used to determine the level of sensitivity of *survivin* mRNA assay in accordance with the method, and *survivin* gene expression by these non-malignant dividing cells was detected, normal cell division being a state that may mimic very early bladder carcinogenesis. Normal human bladder cells sloughed into the urine in the bladder were negative for *survivin* expression. Housekeeping gene *β-actin*-specific mRNAs were detected in all collected samples (data not shown). Thus, detecting *survivin* mRNA transcript in accordance with the inventive methods provides a sensitive, specific assay diagnostic for the presence of neoplasms.

Table 1. *Survivin* mRNA in Urine Sediment *

	cont(-)	cont(+)	cyto(+)	cyto(A)	cyto(neg)
PCR(+)/total	0/6	9/9	2/2	0/3	0/18

*cont(-) = normal; cont(+) = neoplastic cell lines; cyto(+) = cytologically (+) for malignancy; cyto(A) = presence of atypical (degenerative [2]; uncertain [1]) cells; cyto(neg) = cytologically negative for malignancy

The foregoing example being illustrative but not an exhaustive description of the embodiments of the present invention, the following claims are presented.

IN THE CLAIMS

1. A method of detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject, comprising:

collecting a sample of a bodily substance containing human nucleic acid, said sample being derived from the human subject;

amplifying a Survivin-encoding mRNA in said sample to form *survivin*-specific amplification products using *survivin*-specific primer(s) selected from the group consisting of

(A) (SEQ. ID. NO.:1), (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), (SEQ. ID. NO.:4), (SEQ. ID. NO.:5), or (SEQ. ID.NO.:6);

(B) a nucleotide sequence complementary to (A);

(C) a *survivin* gene-specific fragment of (A) or (B) at least 15 nucleotides long; and

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end; and

detecting the presence or absence of expression of a human *survivin* gene in said bodily substance by analyzing the amplification products, wherein the presence of *survivin*-specific amplification products is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the human subject.

2. The method of Claim 1, wherein the substance is urine, blood, feces, semen, prostatic fluid, saliva, gastric juice, mucus, synovial fluid, pleural effusion, peritoneal effusion, pericardial effusion, lymph, cerebro-spinal fluid, skin, hair root, vascular epithelium, oral epithelium, vaginal epithelium, cervical epithelium, uterine epithelium, intestinal epithelium, bronchial epithelium, esophageal epithelium, or mesothelium.

3. The method of Claim 1, wherein the bodily substance is a cellular material.

4. The method of Claim 3,wherein the cellular material is derived from the human subject's kidney, bladder, ureter, urethra, brain, thyroid, parotid gland, submaxillary gland, sublingual gland, lymph node, bone, cartilage, lung, mediastinum, breast, uterus, ovary, testis, prostate, cervix uteri, endometrium, pancreas, liver, spleen, adrenal, esophagus, stomach, or intestine.

5. The method of Claim 1, wherein the neoplastic growth is a carcinoma, sarcoma, lymphoma, mesothelioma, melanoma, glioma, nephroblastoma, glioblastoma, oligodendroglioma, astrocytoma, ependymoma, primitive neuroectodermal tumor, atypical meningioma, malignant meningioma, or

neuroblastoma.

6. The method of Claim 1, wherein the hyperplastic and/or cytologically dysplastic cellular growth or proliferation is benign prostatic hyperplasia/dysplasia or cervical hyperplasia/dysplasia.

7. The method of Claim 1, wherein a *survivin* gene-specific oligonucleotide probe is used in analyzing the amplification products.

8. The method of Claim 7, wherein said oligonucleotide primer and/or probe is labeled with a fluorescent dye.

9. The method of Claim 1, wherein detecting the presence or absence of expression of a human *survivin* gene further comprises comparing the level of survivin expression in said bodily substance relative to the expression of a housekeeping gene, wherein overexpression of the product of the *survivin* gene is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the human subject.

10. The method of Claim 9, wherein the housekeeping gene is a gene encoding β-actin, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, or phosphoglycerate kinase.

11. The method of Claim 7, wherein said oligonucleotide probe comprises:

(A) (SEQ. ID. NO.:1), (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), (SEQ. ID. NO.:4), (SEQ. ID. NO.:5), or (SEQ. ID. NO.:6);

(B) a nucleotide sequence complementary to (A);

(C) a *survivin* gene-specific fragment of (A) or (B) at least 15 nucleotides long; or

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end.

12. A method of detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the urinary tract of a human subject, comprising:

collecting a urine sample from a human subject;

amplifying a Survivin-encoding mRNA in said sample to form *survivin*-specific amplification products using *survivin*-specific primer(s) selected from the group consisting of

(A) (SEQ. ID. NO.:1), (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), (SEQ. ID. NO.:4), (SEQ.

ID. NO.:5), or (SEQ. ID. NO.:6);

(B) a nucleotide sequence complementary to (A);

(C) a *survivin* gene-specific fragment of (A) or (B) at least 15 nucleotides long; and

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end;and

detecting the level of expression of a nucleic acid or protein product of a human *survivin* gene in said urine sample by analyzing the amplification products, relative to the expression of a housekeeping gene, wherein overexpression of the product of the *survivin* gene relative to expression of the housekeeping gene is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in the subject's urinary tract.

13. The method of Claim 12, wherein the neoplastic growth is a malignant neoplasm.

14. The method of Claim 13 wherein the malignant neoplasm is a renal cell carcinoma, bladder carcinoma, nephroblastoma, ureteral tumor, or renal pelvic tumor.

15. The method of Claim 13, wherein the malignant neoplasm is a transitional cell cancer of the renal pelvis.

16. The method of Claim 12, wherein the neoplastic growth is a non-malignant tumor.

17. The method of Claim 16, wherein the non-malignant tumor is an angiomyolipoma, mesoblastic nephroma, or premalignant renal oncocytoma.

18. The method of Claim 12, wherein the housekeeping gene is a gene encoding β-actin, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, or phosphoglyceratekinase.

19. The method of Claim 12, wherein a *survivin* gene-specific oligonucleotide probe is used in analyzing the amplification products.

20. The method of Claim 19, wherein said oligonucleotide primer or probe is labeled with a fluorescent dye.

21. The method of Claim 19, wherein said oligonucleotide probe comprises:

(A) (SEQ. ID. NO.:1), (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), (SEQ. ID. NO.:4), (SEQ. ID. NO.:5), or (SEQ. ID. NO.:6);

(B) a nucleotide sequence complementary to (A);

(C) a *survivin* gene-specific fragment of (A) or (B) at least 15 nucleotides long; or

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end.

22. A *survivin* gene-specific oligonucleotide primer or probe, comprising:

(A) (SEQ. ID. NO.:1), (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), (SEQ. ID. NO.:4), (SEQ. ID. NO.:5), or (SEQ. ID. NO.:6);

(B) a nucleotide sequence complementary to (A);

(C) a *survivin* gene-specific fragment of (A) or (B) at least 15 nucleotides long; or

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end.

23. An oligonucleotide primer set for amplifying a *survivin* gene-specific nucleic acid segment, comprising at least a forward primer and at least a reverse primer, wherein the forward primer is a nucleic acid comprising:

(A) (SEQ. ID. NO.:1) or (SEQ. ID. NO.:4);

(B) a nucleotide sequence complementary to any of (A);

(C) a gene-specific fragment of (A) or (B) at least 15 nucleotides long; or

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end; and

a reverse primer comprising:

(E) (SEQ. ID. NO.:2), (SEQ. ID. NO.:3), or (SEQ. ID. NO.:5);

(F) a nucleotide sequence complementary to any of (E);

(G) a *survivin* gene-specific fragment of (E) or (F) at least 15 nucleotides long; or

(H) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (E) or (F) at its 5' or 3' end.

24. An oligonucleotide primer set for amplifying a *survivin* gene-specific nucleic acid segment, comprising at least a forward primer and at least a reverse primer, wherein the forward primer is a nucleic acid comprising:

(A) (SEQ. ID. NO.:6);

(B) a nucleotide sequence complementary to any of (A);

(C) a gene-specific fragment of (A) or (B) at least 15 nucleotides long; or

(D) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (A) or (B) at its 5' or 3' end; and

a reverse primer comprising:

(E) (SEQ. ID. NO.:2) or (SEQ. ID. NO.:5);

(F) a nucleotide sequence complementary to any of (E);

(G) a *survivin* gene-specific fragment of (E) or (F) at least 15 nucleotides long; or

(H) a *survivin* gene-specific nucleotide sequence overlapping at 5 or more contiguous nucleotide positions any sequence of (E) or (F) at its 5' or 3' end.

25. A diagnostic kit for diagnosing a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject, said kit comprising the oligonucleotide primer set of Claim 23 or Claim 24; and

instructions for using the primer set in diagnosing a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject.

26. A diagnostic kit for diagnosing a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject, said kit comprising at least one of the oligonucleotide primers or probes of Claim 22; and

instructions for using the primer(s) or probe(s) in diagnosing a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject.

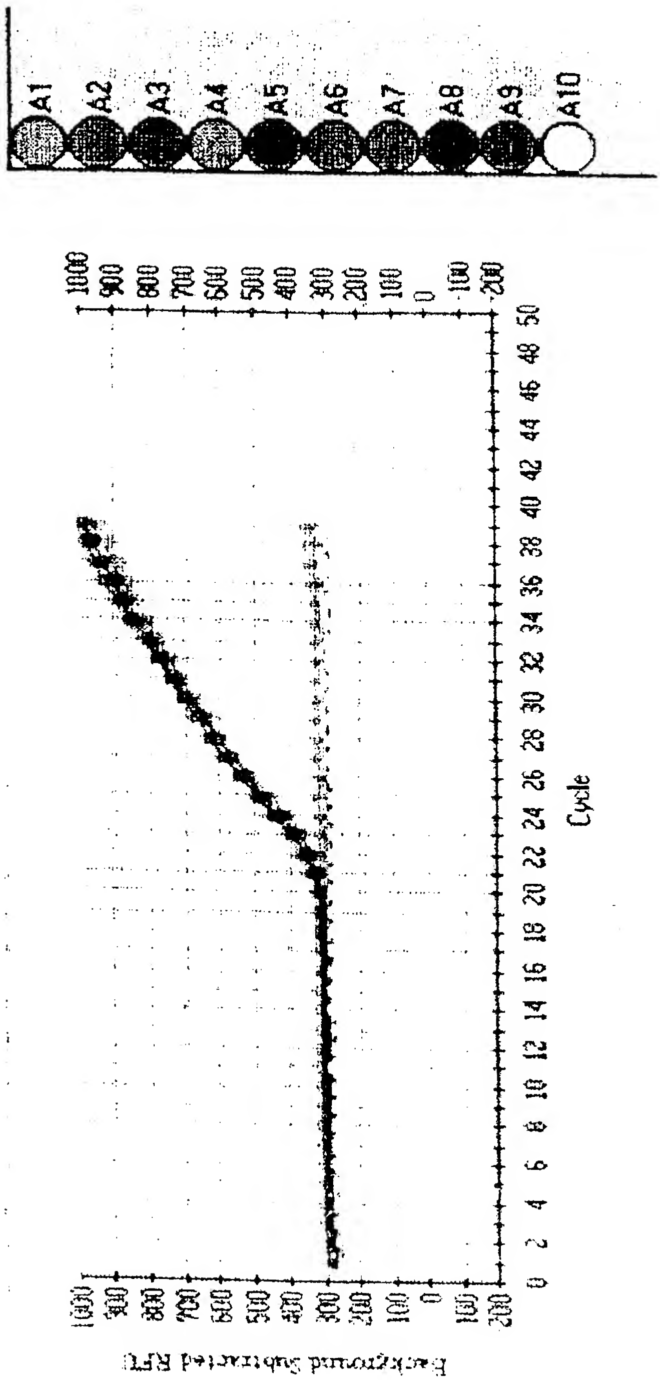

Fig. 1

SEQUENCE LISTING

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W. Stephen Nichols (Inventor)
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<120> Method of Detecting Neoplastic
Hyperplastic, Cytologically Atypical And/Or Premalignant
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<213> Homo sapiens

<400> 6
ccgaggctgg cttcatccac tgc 23

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: METHOD OF DETECTING NEOPLASTIC, HYPERPLASTIC, CYTOLOGICALLY DYSPLASTIC AND/OR PREMALIGNANT CELLULAR GROWTH OR PROLIFERATION

(57) Abstract: Disclosed is a method of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation in a human subject that involves collecting a sample from the human subject of a bodily substance, such as urine, blood, semen, saliva, mucus, feces, or cellular material, which contains the human subject's nucleic acid or protein. Expression and/or overexpression of nucleic acid or protein products of *survivin* gene, detected in the bodily substance, is diagnostic for the presence of neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation. Detection is through amplification by molecular biological techniques of *survivin*-specific nucleic acids and analysis of the amplification products, or by immunochemical assay for Survivin protein. Embodiments of the method are disclosed for detecting a neoplastic, hyperplastic, cytologically dysplastic and/or premalignant cellular growth or proliferation of the human urinary tract, such as a urinary tract neoplasm. Also disclosed are *survivin* gene-specific primers and probes, primer sets, and diagnostic kits containing them.

INTERNATIONAL SEARCH REPORT

Interr Application No
PCT/US 01/01956

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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